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City-level SARS-CoV-2 sewage surveillance

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ABSTRACT

The COVID-19 pandemic created a global crisis impacting not only healthcare systems, but also economics and society. Therefore, it is important to find novel methods for monitoring disease activity. Recent data have indicated that fecal shedding of SARS-CoV-2 is common, and that viral RNA can be detected in wastewater. This suggests that wastewater monitoring is a potentially efficient tool for both epidemiological surveillance, and early warning for SARS-CoV-2 circulation at the population level. In this study we sampled an urban wastewater infrastructure in the city of Ashkelon (150,000 population), Israel, during the end of the first COVID-19 wave in May 2020 when the number of infections seemed to be waning. We were able to show varying presence of SARS-CoV-2 RNA in wastewater from several locations in the city during two sampling periods, before the resurgence was clinically apparent. This was expressed with a new index, Normalized Viral Load (NVL) which can be used in different area scales to define levels of virus activity such as red (high) or green (no), and to follow morbidity in the population at the tested area. The rise in viral load between the two sampling periods (one week apart) indicated an increase in morbidity that was evident two weeks to a month later in the population. Thus, this methodology may provide an early indication for SARS-CoV-2 infection outbreak in a population before an outbreak is clinically apparent.

1. Introduction

SARS-CoV-2 emerged during late 2019 and resulted in an ongoing pandemic that to date is difficult to eradicate. Ascertaining the magnitude of the outbreak is possible when taking into consideration infected symptomatic as well as asymptomatic cases that may unknowingly distribute the virus among the population. Clinical testing capability

that covers vast populations relies on voluntary response and high financial means. These tests are crucial factors for pandemic containment. In addition prolonged incubation periods, and the number of asymptomatic individuals make it difficult to cut the chains of infections (Larsen and Wigginton, 2020). Recent reports have shown that SARS-CoV-2 is shed through human stool (Holshue et al., 2020) and can be detected in wastewater (Thompson et al., 2020), as evident from

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worldwide studies (Wu et al., 2020a), including from the Netherlands (Medema et al., 2020), Australia (Ahmed et al., 2020), USA (Wu et al., 2020b), Germany (Westhaus et al., 2021), Italy (La Rosa et al., 2020a), France (Trottier et al., 2020) and Israel (Bar-Or et al., 2020). These studies rely on wastewater treatment plant (WWTP) samples that can provide geographically large-scale indications of SARS-CoV-2 presence. A recent study by Prado et al., 2020 (Prado et al., 2020), demonstrate the potential for identifying COVID-19 hotspots when sampling sewer networks within the city.

In light of this, monitoring wastewater systems can offer an efficient tool for surveillance of this infectious disease, providing an early warning system. Indeed this strategy has been used for the past decades for monitoring infectious diseases such as Polio (Berchenko et al., 2017), as well as monitoring antibiotic resistance (Hendriksen et al., 2019) and the presence of other microbial pathogens (García-Aljaro et al., 2019). Such a tool can thus provide a more reliable picture regarding population morbidity than clinical diagnostic tests and can be used to determine policy, with regards to dispersion of medical resources. It can be used to assess strategic places such as nursing homes, educational institutions, prisons, military bases, vital industrial plants and more. In addition, the development of such a monitoring platform will also make it possible to identify the emergence of new pathogenic mutations in the population as needed (Lodder and de RodaHusman, 2020).

Therefore, environmental surveillance of SARS-CoV-2 in sewage may

provide a reliable monitoring tool for assessing disease activity and resurgence. This is dependent upon many variables including the sewer system topology, population and usage (Petala et al., 2021). Current review of relevant publications regarding environmental surveillance demonstrates that there is no common concentration method, but different techniques such as, PEG precipitation, ultracentrifugation, electronegative membrane, ultrafiltration and so on have been suggested. As the reported recovery efficiency of these methods are not consistent and differ from sample to sample (Collivignarelli et al., 2020; La Rosa et al., 2020b), there is a need for further research into a way to simplify the methodology that will be consistent, easy to conduct and replicate.

In the present study, composite sampling was carried out from a city (Ashkelon) central wastewater treatment facility (WWTP), as well as from the city neighborhood sewage drainage basins and their cross-section with the main sewer pipeline leading to the WWTP. This design was aimed at developing an urban-sewer sampling strategy for SARS-CoV-2 detection at the city level. Direct RNA extraction from the sewage samples followed immediately by RT-qPCR resulted in faster results thus potentially allowing integration with many clinical labs as a standard routine for epidemiological monitoring. Monitoring of differentially populated areas will enable geographical localization of COVID-19 outbreaks or hotspots.

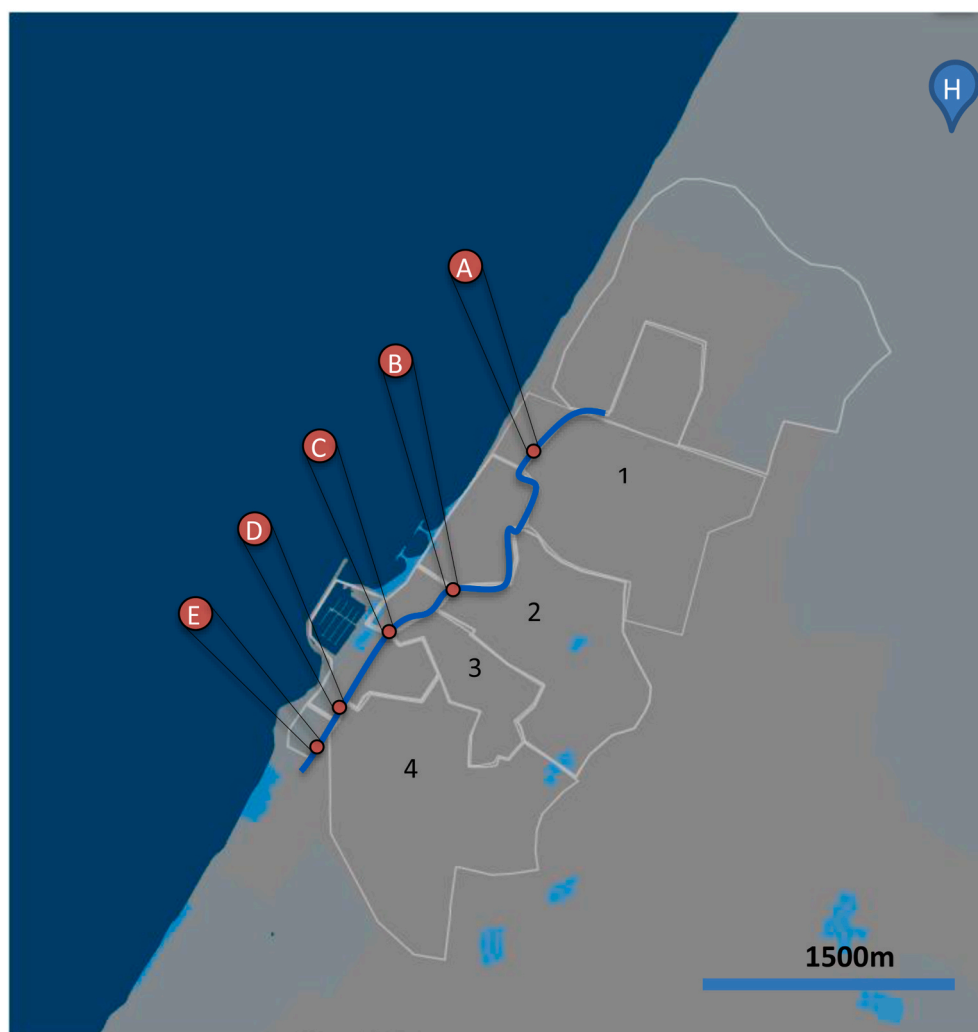


Fig. 1. Deployment of sampling units in the city of Ashkelon. Numbers 1 to 4 present sampling points for the different neighborhoods in the city, defined in the grey line. Letters A to E present sampling points along the city main sewage stream (blue line) leading to the waste water treatment plant (WWTP), point H at the top. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2. Materials and methods

2.1. Sampling

The study was performed in the city of Ashkelon, a Southern coastal city in Israel having a population of approximately 150,000 inhabitants. Ten continuous sewage-sampling units were installed in Ashkelon's wastewater system at nine select sewer manholes and one at the inlet of the wastewater treatment plant (WWTP). The automated composite samplers were activated during two sampling campaigns, the first during 17–19 of May 2020 and the second on the May 25, 2020. Sampling was carried out for 8 h, from 6:00–14:00 or 07:00–15:00 at each point. The sampler unit was composed of a flow rate sensor. The water height was measured to calculate the cross-section area of the flow (using Manning Formula). At initial assembling of the unit in the manhole, the velocity of the flow was measured to validate measurement accuracy. The chosen sampling areas included several neighborhoods along the coast of Ashkelon (tagged as 1 to 4 in Fig. 1). The sampling points included only domestic wastewater. They were connected to a central sewer line that was also sampled in designated sewer junction manholes (A to E in Fig. 1). This central sewer line eventually drains into the municipal WWTP (designated as point H) that treats the city wastewater. For neighborhoods 1 to 4, the estimated population sizes were 13,500; 5600; 2500 and 12,500 respectively, based on national census data (the Central Bureau of Statistics, https://www.gov.il/en/departments/central_bureau_of_statistics).

2.2. Sample handling, RNA extraction and analysis

Collected raw sewage samples (1 L composite sample) were immediately transferred to the lab under chilled conditions. The samples were kept up to 2 weeks at -20°C until processed. Appropriate protective gear (masks, gloves, and coveralls) was used until the RNA extraction step. The sewage samples were opened under a combined BSL-2 biological and chemical hood. The initial RNA extraction step (lysis buffer) took place under the hood. Upon nucleotide acid extraction, the samples were then taken out of the hood and only regular protection (lab coat,

amplification was executed using Step One Plus real-time PCR system (Applied Biosystems, Thermo Scientific). In parallel to the N1 test, each RNA sample was spiked with N gene plasmid in known concentrations to rule out any amplification inhibition effect. A second quality control step was carried out by adding known amount of MS2 phage copies to the lysis buffer step as process control for RNA extraction. For each qPCR plate setup, both quality controls were tested in the presence of dH_2O as well as in each of the sewage RNA samples. Ct values were compared to determine inhibition affect (no greater than three Ct cycles). The analytical LOD (limit of detection) for N gene target was determined to be < 10 target copies per qPCR reaction. For more details, full primers, and probe list, see section S2 and Table S1 in supplement information.

2.3. SARS-CoV-2 RNA copy number calculation

SARS-CoV-2 RNA copy number per 1 L was calculated using the standard curve equation (Fig. S1). A new index, namely Normalized Viral Load (NVL) was defined. It expresses the estimated number of SARS-CoV-2 RNA copies per 1000 people. It is based on quantification of the cumulative number of copies during several hours of composite wastewater sample collection (8 h in this study) in a specific location, within a steady timeframe of a day (see Equation (1)). Additional normalization was based on the measured Total Nitrogen (TN) concentration using Equation (2), where 8.5 was considered as typical specific TN load (Tsuzuki, 2006; Van der Hoek et al., 2018) (g N per person per day). Furthermore, we calculated Pearson correlation coefficient value between the normalized values by population size (equation (1)) versus normalization by TN values (equation (2)).

$$\text{Normalized Viral Load} \left(\frac{\#}{1000 \text{ person}} \right) = \frac{\text{RNA copy number} \left(\frac{\#}{\text{L}} \right) * \text{Cumulative sampling flow (L)}}{\text{Population size} / 1000} \quad \text{Equation 1}$$

$$\text{Normalized Viral Load by TN} \left(\frac{\#}{\text{person}} \right) = \frac{\text{RNA copy number} \left(\frac{\#}{\text{L}} \right) * \text{Cumulative sampling flow (L)}}{\text{Average daily flow rate} \left(\frac{\text{L}}{\text{day}} \right) * \frac{\text{TN} \left(\frac{\text{g}}{\text{L}} \right)}{8.5 \frac{\text{g N}}{\text{person day}}}} \quad \text{Equation 2}$$

face masks and gloves) was needed for downstream steps. For sample disposal, all sewage samples were treated overnight with 2% Sodium hypochlorite and autoclaved for 40 min at 121°C . After treatment, the containers were disposed as regular waste. Prior to subsampling, the bottles were shaken vigorously and left to rest for 10 min. Subsamples of 0.2 mL raw sewage (in duplicate) were then transferred directly into NucleoSpin RNA lysis buffer for RNA extraction (Macherey Nagel, Germany). RNA was eluted with 50 μL of RNase free water and kept at -80°C . SARS-CoV-2 detection was executed using One Step PrimeScript III RT-qPCR mix with no changes in manufacture protocol (RR600 TAKARA, Japan). The reaction mixture contained the CDC's primers and probe N1¹⁴ together with five μL of eluted RNA and final volume of 20 μL . The presented Ct values are the average of the two replicates. For creating a standard curve, we used a plasmid containing the full SARS-CoV-2 N gene sequence isolated from Wuhan-Hu-1 (GenBank: NC_045512.2). We prepared standard curves of plasmid log copy number versus Ct value using serial dilutions of the plasmid. We then performed linear regression between the log copy number and the Ct values from the RT-qPCR results. Using the linear equation, we calculated copy number of N1 gene in sewage samples reported in this study. RT-qPCR

3. Results and discussion

The city of Ashkelon was chosen for surveillance of SARS-CoV-2 in sewage due to a relatively low COVID-19 prevalence during the time of the study following a national lockdown period during April 2020 resulting in life gradually returning to normal at the beginning of May. Ashkelon is a metropolitan area with approximately 150,000 residents. In this study, the urban sewage sampling system design was composed from 10 continuous sewage-sampling units. During May 2020 we deployed continuous sampling devices in nine major sewer manholes in the city's wastewater system and an additional device in the city wastewater treatment plant (WWTP). As shown in Fig. 1, numbers 1 to 4 present sewage drainage basins manholes sampling points for each neighborhood separately. Letters A to E present the neighborhood's cross-section outlet to the main sewer pipeline sampling points leading to the city WWTP (point H). Direct RNA extraction for SARS-CoV-2 RNA detection and chemical analysis of these samples were performed from

Table 1
Results for Normalized viral RNA copy number calculated different measured parameters.

Sample name	Sample date	TN (g/L)	Flow (m ³ /hr)	NCOV- N1 (Ct)	N1 (RNA concentration per L)	Population	Normalized Viral Load by TN (copy number per person)	NVL (RNA copy number per day per 1000person)
H	25.05	0.092	1491.39	35.22	9.02E+06	150,000	2.23E+06	5.74E+12
E	25.05	0.099	590.64	ND		48,500		
D	25.05	0.094	497.65	35.89	5.67E+06	35,800	1.37E+06	5.04E+12
C	25.05	0.103	390.94	ND		32,800		
B	25.05	0.076	322.74	35.3	8.53E+06	27,000	2.53E+06	6.53E+12
A	25.05	0.101	149.70	33.18	3.71E+07	12,800	8.34E+06	2.77E+13
4	25.05	0.089	93.00	34.86	1.16E+07	12,500	2.96E+06	5.51E+12
3	25.05	0.084	106.71	36.66	3.32E+06	2500	9.02E+05	9.08E+12
2	25.05	0.123	68.20	35.24	8.89E+06	5600	1.64E+06	6.93E+12
H	19.05	0.091	1525.94	ND		150,000		
E	19.05	0.092	485.56	ND		48,500		
D	18.05	0.097	507.61	ND		35,800		
C	19.05	0.094	401.34	ND		32,800		
B	18.05	0.060	225.00	ND		27,000		
A	17.05	0.096	64.52	34.9	1.13E+07	12,800	2.67E+06	3.63E+12
4	18.05	0.098	-45.43	ND		12,500		
3	19.05	0.078	108.62	ND		2500		
2	19.05	0.099	167.63	ND		5600		
1	18.05	0.097	160.48	34.31	1.69E+07	13,500	3.95E+06	1.29E+13

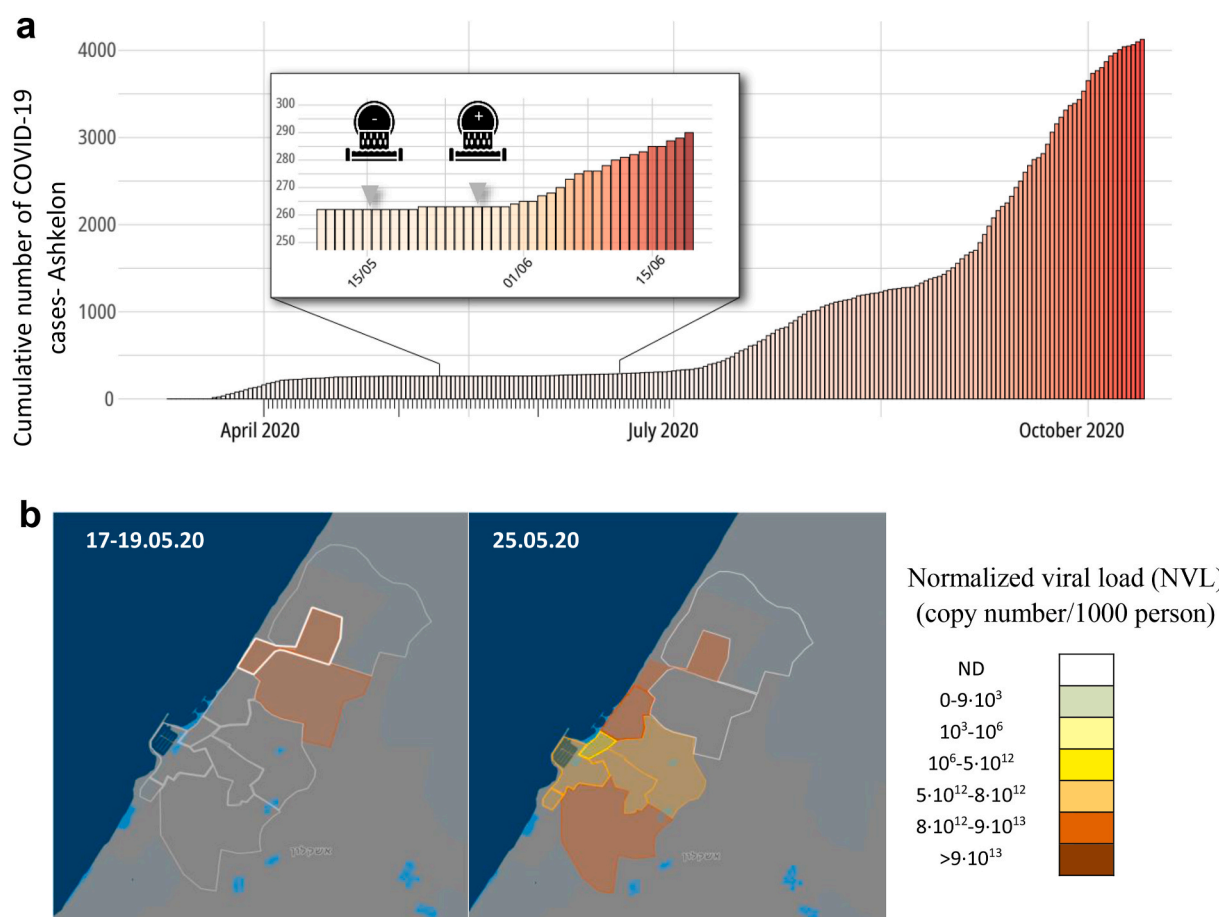


Fig. 2. (a) Cumulative COVID-19 cases in Ashkelon during March until August 2020. Black dash line presents the beginning of the second COVID-19 outbreak (reported by health care diagnostics). Arrows in the zoomed-in box, represent the sampling campaign dates in May 2020. (b) Normalized SARS-CoV-2 viral loads (NVL) calculated from wastewater collection campaign from different geographic locations at the city of Ashkelon. Numbers 1 to 4 present sampling points for the different neighborhoods in the city, defined in the grey line. Letters A to E present sampling points along the main city main sewage stream (blue line) leading to the wastewater treatment plant (WWTP). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

each sewage sample, as were the TOC, TN, and pH measurements (see Table 1).

In this study, we carried out two sampling campaigns during May 2020, a period with low prevalence of COVID-19 in all of Israel. Despite

the fact that during this time no new COVID-19 positive cases had been reported in Ashkelon by the Ministry of Health (Fig. 2a and zoom in box), we found traces of the SARS-CoV-2 RNA in sewage originating from the different sampling manholes in the city (Fig. 2b). Fig. 2a

presents the accumulation of active COVID-19 cases in Ashkelon as quantified by healthcare diagnostics and reported by the Ministry of Health. The arrows in the figure indicate the two sampling efforts during May 2020.

In order to compare viral RNA concentration between different cities or neighborhoods, we performed a normalization of SARS-CoV-2 RNA copy number using two equations Equations (1) and (2). Equation (1) was based on measured flow rate at each of the sampling points and the population size (Table 1). In Equation (2), we estimated population size using the measured TN value and a literature specific TN load (g N per person per day). The two normalizations methods (Table 1) were found to be highly correlated, with Pearson correlation coefficient of 0.89 (Fig. S3), meaning population estimation by TN is possible. A study by Petala et al. (2021), demonstrated a correlation between dissolve oxygen and viral RNA copies, suggesting that oxidation damage viral detection. More research on additional sewage parameters, both chemical and biological, is necessary for better understanding viral dynamics. Considering that this study measured parameters availability, we choose Normalized Viral Load (NVL) index for comparison of SARS-CoV-2 RNA detection between the study different sampling locations (Fig. 2b). This index can be fit to various scales such as a street, a quarter, a small town, or a large city. Sampling location, duration, and method (preferably composite) should be unified to enable reliable use of the NVL. If the sampling is extended to 24 h, then the NVL can be expressed as number of SARS-CoV-2 RNA copies per 1000 people. Nevertheless, even sampling of several hours during a steady timeframe serves as a reliable relative index to be assessed chronologically. This index can assist to define levels of virus traces in a semi-quantitative fashion (e.g., using a 'traffic-light' scheme), and to follow morbidity of the population in the tested area.

During the first sampling campaign, two sampling locations A and 1, (located in the north of the city) were found to be positive for SARS-CoV-2 with normalized viral load (NVL) of $3.36 \cdot 10^{12}$ and $1.29 \cdot 10^{13}$ (copy number/1000 person) respectively. The remaining sampling points were negative (Not Detected, ND) including point H, which is the inlet of the WWTP. It is important to note that the samples were kept between one to two weeks in -20°C conditions until processing. Results in Supplementary information (Fig. S2) reveal that thawing the samples leads to one log lost in signal detection. Thus, this study's observations remain valid and support the study conclusions. Considering these results, we recommend keeping the samples at 4°C and processing them within a week. The fact that negative detection was observed in point H, while two samples out of the ten were positive, suggests a dilution effect at the main sewer pipeline and at the WWTP. This dilution effect in the upstream sewer system suggests a low sensitivity of the method when sampling is applied only to the WWTP or to larger geographical areas, especially when the morbidity is low and/or highly localized. Moreover, direct RNA extraction provided the possibility for quick identification of the north area of the city (location 1 and A) as potentially morbidity center. Numerous studies (Medema et al., 2020; Ahmed et al., 2020; Westhaus et al., 2021; La Rosa et al., 2020a; Trottier et al., 2020; Santiso-Bellón et al., 2020) have shown that the surveillance of different viruses in samples taken from wastewater treatment plants is useful for studies of population transmission and epidemiology. These studies concentrated the nucleic acids found in sewage samples using different methods, such as ultracentrifugation, PEG precipitation and electro-negative membrane. Although concentration treatments may provide overall better detection sensitivity, here we present an alternative direct RNA extraction for SARS-CoV-2 detection based on city manhole sampling design for quick surveillance and alert. This method is also cost-effective and can easily integrate in many clinical labs as a standard routine for epidemiology monitoring.

In the second sampling campaign, all sampling points were positive, with NVL values of between $5.04 \cdot 10^{12}$ and $2.77 \cdot 10^{13}$ (copy number/1000 person). Unfortunately, due to technical problem point 1 sample is missing from the second campaign. Interestingly, the viral load in point

A increased by an order of magnitude from NVL of $3.36 \cdot 10^{12}$ to $2.77 \cdot 10^{13}$ (copy number/1000 person) between the two sampling campaigns. Fig. 2b demonstrates, by color code, the NVL in each of the sampled manholes, recording the profound changes in viral load that occurred during the two sampling campaigns. While two time points are not enough to establish a trend, it is important to point out that we detected a positive SARS-CoV-2 RNA signals in the city sewage system about one week prior to July COVID-19 initial outbreak of the second wave of in the city (seen in Fig. 2a). When focusing on the May sampling dates (Fig. 2a), one can observe that there was no relationship to the reported COVID-19 cases. The low morbidity in the city reported during May, particularly at the end of the month, might have been an under-estimation of COVID-19 cases due to limited testing efforts and due to untested asymptomatic cases (as per testing guidelines at the time of study). The presence of SARS-CoV-2 RNA in the city sewage, despite the low prevalence reported in May, indicates the sensitivity and importance of this method for early detection and for geographic localization of potentially new morbidity center.

Indeed, a resurgence in COVID-19 cases became evident in the city's population in June–July 2020 a month following the wastewater sampling. Moreover, we would have expected that low morbidity and no new cases in the first sampling point together with the fact that we did no concentration process prior to sewage RNA extraction, will have resulted in No Detection result following RT-qPCR test. From looking at the calibration curve (Fig. S1), the limit of detection is 10 copies per reaction and $5 \cdot 10^5$ copies per L raw sewage sample. The positive SARS-CoV-2 signal implies that despite lack of reported clinical cases during that time, we were able to show that this method can be sensitive to overcome the dilution effect with no additional sample processing. However, additional concentration procedure can be employed in future research and application.

4. Conclusions

Calculation of viral RNA load index normalized to population size and flow rate value enabled us to compare RNA copy number between different sampling points (Fig. 2b). Several studies have been published regarding morbidity estimation based on sewage viral load detection (Ahmed et al., 2020; Hart and Halden, 2020). It seems that to date, it is not possible to directly translate viral RNA concentration detected in wastewater to morbidity level in a population. There is a great variability in viral RNA shedding with time and among infected people making it hard for estimation. There is also variability in the sewer chemical characteristics between communities, size, rainwater and industrial wastewater. However, considering the benefits of developing an early warning system that does not depend on population compliance and trends in population COVID-19 surveillance, the use of wastewater epidemiology is a great value.

Understanding the dynamics of SARS-CoV-2 in human excreta could lead to efficient monitoring and surveillance of this virus as well as to underpin environmental surveillance (Mohan et al., 2021). Our results also demonstrate the need for inner-city sewer sampling efforts to overcome dilution effect that can eventually affect the sensitivity and validity of this surveillance method. Such an inner-city sewer study may assist in geographical localization of disease hotspots in a quick and cost-effective manner. Multiple sampling efforts within a city could allow assessment of virus spread in a given area and assess the proportion of areas with virus activity in a city, thus predicting imminent resurgences.

In conclusion, we present a proof-of-concept study demonstrating the feasibility of SARS-CoV-2 RNA detection in raw sewage originating from the city sewer system that reflects virus circulation in the assessed area. The method can be implemented to many clinical labs as a standard routine for epidemiology monitoring, as it does not require high loads and complicated lab procedures for sample concentration. This approach should be further studied and validated as an early warning for

SARS-CoV-2 resurgence in urban settings and as a national decision support tool.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2021.131194>.

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